Achatina fulica mucous improves cell viability and increases collagen deposition in UVB-irradiated human fibroblast culture

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Abstract

Introduction: Ultraviolet radiation induces skin photoaging by increasing matrix metalloproteinase-1 (MMP-1). MMP-1 degrades type I and III collagen that comprise the dermal connective tissue. *Achatina fulica* mucous (AFM) is a natural remedy that has protective effects on fibroblasts and collagen.

Objective: To investigate the effects of AFM on cell viability and collagen deposition in UVB-irradiated human fibroblast culture.

Methods: The mucous was extracted from 50 *Achatina fulica* snails that were stimulated by a 5-10 Volt electricity shock for 30-60 seconds and converted into powder by the freeze-drying process. The human dermal fibroblast culture was divided into six groups: group 1 were normal fibroblasts without UVB irradiation as normal control, groups 2-5 consisted of 100 mJ/cm² UVB-irradiated fibroblasts. Group 2 had no treatment as negative control, group 3 was treated by PRP 10% as positive control group and groups 4-6 were treated by various concentrations of AFM (3.9; 15.625 and 62.5 µg/mL). At the end of the experiment, the proliferation was assessed with MTT assay, furthermore collagen deposition was measured by Sirius red assay. Real Time-PCR (RT-PCR) was performed to quantify Coll I, Coll III and MMP-1 mRNA expression, then to measured COL I/COL III ratio.

Results: UVB induced significant lower viability, upregulated MMP-1 and downregulated COL I and COL III mRNA expressions. Meanwhile AFM treated groups demonstrated higher cell viability with downregulation of MMP-1 and upregulation of COL I and COL III mRNA expressions. The ratio of COL I/ III expression was significantly ($p < 0.05$) lower in the AFM treated groups compared to the UVB group. Among AFM treated groups, administration of 62.5 µg/mL AFM represented the best result.

Conclusion: AFM may ameliorate viability of UVB-irradiated human fibroblast culture which associates with downregulating MMP-1, upregulating COL I and Col III, and reducing COL I/III ratio.

Key Words: UVB-irradiated human fibroblast culture; *Achatina fulica*; Viability; MMP-1; Collagen

Introduction

Ultraviolet (UV) radiation is the main external factor leading to skin aging. This effect of UV radiation is related to the exposure to specific wavelengths of sunlight. Based on the wavelength, there are several types of UV, which are UVA, UVB, and UVC^[1]. UVC radiation with 200-290 nm in wavelength is reflected by the ozone layer, so its impact on the skin is not significant. The shorter wavelengths have greater impact. UVB with a wavelength of 290-320 nm provides more energy than UVA which has a wavelength of 320-400 nm. UVB is able to penetrate the epidermis and dermis. UVB exposure is linked to a higher non-melanoma skin cancer (NMSC) risk compared to UVA. Increasing UVB radiation on the earth induces inflammatory responses, apoptosis, and subsequent skin damage, such as photoaging^[2].

The characteristics of photoaging are wrinkles, loss of elasticity, dryness, frailty, rough skin texture, telangiectasias and pigmentation disorders that indicate structural and functional alteration of extracellular matrix (ECM)^[3]. The main component

of ECM is collagen that forms the basic framework of connective tissue. The dermal collagen particularly consists of collagen type I^[4]. The type I collagen is a heterotrimer molecule characterized by the triple helix with three related α chains. It indicates mature collagen, forms thick fibers and maintains high tensile strength. The least amount of dermal collagen is collagen type III consisting of a supercoiled $\alpha 1$ (III) chains that forms a homotrimer molecule^[5]. Collagen III has thin fibers with low tensile strength and is also called embryonic or immature collagen^[6]. As age increases, collagen decreases by about 1% / year. Decreased type I and III collagen along with an elevation of the type I/III collagen ratio are indicators of photoaging skin^[7]. Collagen degradation is caused by MMP-1 due to increasing reactive oxygen stress (ROS)-induced by UVB exposure^[8].

Current research has focused to discover anti-aging agents from natural ingredients, plant products, and herbal extracts^[9]. *Achatina fulica* mucous (AFM) is a natural remedy containing anti-aging agents that have been used in ancient medicine. Previous study revealed that AFM contains *Achasin*, a broad spectrum antibiotic and anti-inflammation agent^[10]. Moreover, AFM also contains

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Achran sulfate which is a glycosaminoglycan^[11], the trace mineral, copper which contributes to collagen synthesis, and vitamins A and E as antioxidants^[12, 13].

Accordingly, this experiment aimed to determine how the AFM, which is rich in antioxidants, anti-inflammation activity, and glycosaminoglycans can be turned into high end-value products. In this paper, we observed the protective effects AFM on UVB-induced fibroblast photoaging by assessing collagen deposition, MMP-1 expression and the altered ratio of type I/III collagen expression.

Materials and Methods

This *in vitro* research with post-test only control group design had obtained approval from the ethics committee of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada (Ref: KE/FK/0682/EC/2018).

Snails mucous and materials

AFM was collected by triggering the mucous secretion from 50 *Achatina fulica* snails using 5-10V electricity shock for 30-60 seconds. The mucous was stored in a sterilized jar before converted into powder by freeze-drying process^[12]. Platelet-rich plasma (PRP) was purchased from the Dermato-Venerology Department of Medicine, Public Health and Nursing Faculty, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Cell Culture

We cultured normal human dermal fibroblasts (NHDFs) to be used for experiments at passages >4 from 11-13 years old boys' foreskins. NHDFs were maintained using Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich Corporation, St. Louis, MO, USA) which were supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 units/ml Penicillin, and 100 µg/ml Streptomycin (Penstrep-Gibco; Invitrogen Corporation, Carlsbad, CA, USA) in the humidity of 5% CO₂ at 37°C. Subculture procedure using trypsin was performed in the fibroblasts. Briefly, after rinsing with phosphate-buffered saline (PBS), cells were detached using trypsin and were cultured with completely fresh growth medium.

UVB irradiation and treatments

UVB irradiation procedure was done in the NHDFs model. After 80-90% confluence, the NHDFs were rinsed with PBS and then exposed to UVB light in fresh PBS-filled wells with a bank of six UVB lamps (Philips UVB TL 40W/12RS; LIPI, Jakarta, Indonesia) at a dose of 100 mJ/cm² for 330 seconds. Then, the NHDFs were rinsed with PBS three times and immediately treated with AFM in 3.9 µg/mL, 15.625 µg/mL and 62.5 µg/mL concentrations. Normal controls consisted of the NHDFs that had no treatment and were not exposed to UVB irradiation, and negative control were NHDFs without any treatment and were exposed to UVB radiation, while positive control was the NHDFs that were exposed to UVB radiation with 10% PRP treatment. Then, the NHDFs were incubated for 72 hours.

NHDFs viability procedure using MTT assay

We used MTT (3-(4, 5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide) assay procedure to examine NHDFs viability by monitoring color changes during reduction of tetrazolium salts. Ten microliters of the MTT reagent were added to every well after 72 hours. Then, the NHDFs were incubated in humidity with atmosphere (5% CO₂ at 37°C) for 2 hours. Next, the NHDFs were viewed under an inverted microscope. When the intracellular purple formazan crystals were clearly visible, the substrate-containing medium was removed, and

100 µL of DMSO were added to each well. The optical density (OD) was read using a microplate absorbance reader (Biorad iMark™, Bio-Rad Laboratories, Singapore) at λ 570 nm.

Measurement of collagen deposition by Sirius red

The Sirius red staining procedure was done to quantify collagen deposition in the NHDFs. The NHDFs were rinsed with PBS. Furthermore, at room temperature NHDFs were fixed with Bouin's solution for 60 minutes. The solvent was eliminated and plates were rinsed in running tap water for 15 minutes. The 96-well plates were air-dried overnight, after that 200 µL Picro-Sirius Red dye (1mg/mL in picric acid) was added to every well for 60 minutes with light shaking. In each well, unbound dye was removed by rinsing thrice with 0.01 N HCl while the bound dye was dissolved with 100 µL 0.5 N NaOH with light shaking at room temperature for 30 minutes. Collagen-dye optical density (OD) was read with a microplate absorbance reader (Biorad iMark™, Bio-Rad Laboratories, Singapore) at λ 540 nm.

Measurement of MMP-1, COL I, and COL III mRNA expresión

Quantitative real-time PCR (qRT-PCR) was conducted to quantify the mRNA expression of MMP-1, COL I, and COL III. The levels of mRNA expression were normalized with GAPDH as the reference gene. Briefly, RNA was extracted from the NHDFs by miRCURY Cell And Plant Isolation Kit (Exiqon; Lot# 32213). cDNA was synthesized using miRCURY LNA Universal RT microRNA PCR Universal cDNA Synthesis Kit II, 8-64 rxns (Exiqon; Lot # 629693). RNA was quantified with a nanodrop using NanoVue Plus spectrophotometer (Biochrom, a division of Harvard Bioscience, Inc., Harvard, USA). Quantitative PCR was conducted using protocol from Applied Biosystem 7500 FAST real-time PCR using Toyobo thunderbird® SYBR® qPCR Mix (cat#QPS-201). The qRT-PCR was done with the following conditions: initial denaturation at 95°C for 2 min, followed by 40 cycles for denaturation at 95°C for 10 sec., annealing at 58°C for 15 sec., and extension at 72°C for 20 sec. The 2-ΔΔCq method was used to assess the mRNA expression of MMP, COL I, and COL III. The following primers were used:

MMP1: forward CTGAAAGTGACTGGGAAACC and reverse GACAACTGAGCCACATCAG,
COL I: forward GTGCTAAAGGTGCCAATGGT and reverse GTGGGGAATGGCAAGCAAAA;
COL III: forward CCAGGAGCTAACGGTCTCAG and reverse CAGGGTTTCCATCTCTTCCA,
GAPDH: forward GGAGCGAGATCCCTCCAAAAT and reverse GGCTGTTGTCATACTTCTCATGG.

Each reaction was run in triplicate.

Statistical Analysis

The results are stated in average ± standard error of measurement (SEM). One-way ANOVA was done to determine any significant differences between groups followed by LSD test for the Post-hoc multiple comparison test. The degree of statistical significance was set at p<0.05. The SPSS 22 was performed to assess statistical analyses (IBM Corporation, Chicago, USA).

Results

AFM increased fibroblast viability

UVB exposure induces cell death in normal human dermal fibroblasts (NDHFs). In order to evaluate the viability of the AFM, the NDHFs were treated with serial concentrations (0.49, 0.98, 1.95,

Figure 1. Fibroblast viability test of AFM in NDHFs. NDHFs were cultured in DMEM until 80-90% confluence and exposed to 100 mJ/cm² ultraviolet B (UVB). Cells were added with or without serial concentrations of AFM for 72 h, and afterward the MTT assay was conducted. All data are counted as the average $\pm$ SEM of triplicate experiments. Normal control (NC) was NDHFs not exposed to UVB irradiation and received no treatment, UVB was NDHFs exposed to UVB irradiation and received no treatment, UVB+PRP was NDHFs exposed to UVB irradiation and received treatment with PRP 10%, and UVB+AF was NDHFs exposed to UVB irradiation and received treatment with various concentrations (μ g/mL) of AFM.

3.90, 7.81, 15.625, 31.25, 62.5, 125, 250, and 500 μ g/mL) of AFM for 72 hours after exposure to UVB (100 mJ/cm²). The viability of NDHFs were quantified using an MTT assay (as described in the methods). The AFM viability increased up to 62.5 μ g/mL concentration but showed slight reduction at 125 μ g/mL concentration (Figure 1). Therefore, three different concentrations (3.9 μ g/mL, 15.625 μ g/mL and 62.5 μ g/mL) of AFM were chosen to test in this study. As a positive control, PRP had a viability of 2 folds higher than UVB as a negative control. Compared to PRP, the AFM viability of NHDF 15.625 μ g/mL was not significantly different ($p > 0.05$) but the

AFM viability of NHDF 62.5 μ g/mL was significantly ($p < 0.05$) higher whereas AFM 3.9 μ g/mL was significantly ($p < 0.05$) lower.

AFM downregulated MMP-1 mRNA expression

UV exposure increases MMP-1 mRNA expression which triggers degradation of fibrillar collagen, especially type I and III collagen in the dermis. To observe the effects of AFM on UVB-mediated mRNA expression of MMP-1, the mRNA levels were quantified by qRT-PCR. After UVB-irradiation, the NDHFs were incubated for

Figure 2. Quantitative PCR (qPCR) examination of MMP-1 mRNA expression. NDHFs were induced with UVB 100mJ/cm² and administered with various concentrations of AFM (3.9 μ g/mL; 15.625 μ g/mL and 62.5 μ g/mL). The value is provided as the mean $\pm$ SEM. * $p < 0.05$ indicates the significant versus the normal control. # $p < 0.05$ indicates the significant versus a UVB. \$ $p < 0.05$ indicates the significant versus PRP. Normal control (NC) was NDHFs not exposed to UVB irradiation and received no treatment, UVB was NDHFs exposed to UVB irradiation and received no treatment, UVB+PRP was NDHFs exposed to UVB irradiation and received treatment with PRP 10%, and UVB+AF was NDHFs exposed to UVB irradiation and received treatment with various concentrations (3.9 μ g/mL; 15.625 μ g/mL and 62.5 μ g/mL) of AFM.

72 hours with 3.9 $\mu\text{g/mL}$, 15.625 $\mu\text{g/mL}$ and 62.5 $\mu\text{g/mL}$ concentrations of AFM. As shown in Figure 2, UVB induction in the UVB group demonstrated significantly higher MMP-1 mRNA expression compared to the normal control group (NC). Significantly lower mRNA expression of MMP-1 was shown in the PRP and AFM treated groups. In the AFM group, we found significant differences between AF62 compared to AF3 and AF15, but there was no significant difference between AF3 and AF15. AFM increased collagen deposition, COL I and COL III mRNA expressions with reduced COL I/III ratio.

The *in vitro* effect of AFM on NHDFs collagen deposition was quantified by Sirius red dye binding and spectrophotometry. Incubation of NHDFs for 72 hours with various concentration of AFM after irradiation with 100 mJ/cm^2 UVB induced an enhancement in the amount of NHDFs collagen deposition (Figure 3A). The red stainings represent collagen deposition. In the UVB group, collagen deposition was lower than the normal control group ($p < 0.05$). Moreover, collagen deposition arrangements were more compact and regular in the AFM groups (Figure 3A). The highest concentration of AFM (62.5 $\mu\text{g/mL}$) exhibited significantly ($p < 0.05$) higher collagen deposition. A confirmatory experiment assessed the type of collagen by qRT-PCR examination of COL I, COL III, and COL I/III ratio (Figure 3B).

Discussion

This experiment highlighted the molecular mechanism of AFM for increasing cell viability and collagen deposition in UVB-induced human fibroblast culture through alteration of the MMP-1, and ratio

of type I/III collagen mRNA expression. UVB irradiation (290–320 nm) leads to increased cell death in NDHFs^[13]. In this study, the NDHFs viability in the UVB group decreased compared to the other groups. This finding indicated that the dose of UVB irradiation was adequate to conduct irradiation procedures in line with the previous study. UVB activates cytokines productions that affect cellular mitosis, apoptosis, and cell death. UVB induces the generation of ROS including superoxide anion (O_2^-) or hydrogen peroxide (H_2O_2)^[14]. In this study, we found an improvement of NDHFs viability in the AFM groups compared to UVB groups (Figure 1). AFM contains vitamin A and E (data not shown) as antioxidants that will directly react with ROS. ROS triggers the expression of pro-inflammatory cytokines production. Interestingly, AFM contains *Achasin* that had been reported to have anti-inflammatory effects on the skin^[15].

Increasing of ROS induced by UVB stimulates the synthesis of MMP-1. ROS activates MAP kinases which initiates activator protein 1 (AP-1). AP-1 performs an important role in the transcriptional regulation of MMP-1 which triggers an enhancement of MMP-1^[8]. MMP-1 is the main protease that is competent to initiate fragmentation of fibrillar collagen in the skin, especially types I and III collagen, while other types of MMP further break down degraded collagen fragments. Collagen is arranged by repeated triple helix that is stabilized by cross linking to other molecules. MMP-1 breaks collagen fibrils at the centers of the triple helix. Therefore, an increase in MMP-1 causes the accumulation of collagen fragments and damages the structure and function of the ECM^[16]. Our study revealed upregulation of MMP-1 mRNA expression after UVB exposure (Figure 2), while AFM treatment

Figure 3. A. Representative pictures of Sirius red staining for collagen deposition. Positive staining was reddish shown. B. Quantitative PCR (qPCR) interpretation of COL I, COL III, and COL I/III ratio mRNA expressions. UVB+AF62 group demonstrated significantly higher COL I and COL III mRNA expressions compared with the NC group. UVB group demonstrated significantly lower expressions of both COL I and COL III mRNA compared with the NC group. * $p < 0.05$ indicates the significant versus the normal control. ** $p < 0.05$ indicates the significant versus a UVB. \$ $p < 0.05$ indicates the significant versus PRP.

downregulates the MMP-1 mRNA expression. Compared to PRP as a positive control, AFM has greater effects in downregulation of MMP-1. Previous study reported that PRP is a potent anti-aging material capable of inhibiting MMP-1 mRNA expression and enhancing collagen synthesis^[17].

Collagen, synthesized by fibroblasts, is the major constituent of ECM. It constitutes the basic structure and covers 70-80% of the connective tissue dry weight. Characteristic of the structure is the repeated triple helix that helps collagen to form cross-links with other molecules to maintain mechanical integrity and tensile strength. Increasing MMP-1 due to UVB irradiation induces fragmented collagen which downregulates new collagen synthesis and collagen deposition^[18]. This previous finding is parallel with the histological appearance in this study in Figure 3A that shows UVB reduced collagen deposition while AFM treatment significantly promoted the collagen deposition in NHDFs, which was evidenced by the Sirius red collagen staining.

Commonly, the collagens found in the skin are collagen type I and III. Type I collagen accounts for approximately 85%-90% from the total fibrillar collagen in the skin. Collagen I is mature collagen, which has thick fibers and high tensile strength. Previous study revealed that type I collagen synthesis decreases in photoaged skin^[19]. Our study confirmed that AFM upregulates COL I mRNA expressions. As shown in Figure 3B, AFM increased type I collagen which was reconfirmed by the quantification of COL I mRNA expression in UVB-induced NHDFs. Increasing COL I mRNA expression indicates increasing type I collagen synthesis.

The lesser amount, approximately 10%, of dermal collagen is type III collagen. It has a particular assignment in early organ development as embryonic collagen. The type III collagen is immature collagen which has a thin bundles and low tensile strength. The number of type III collagen is highest in fetal development and then decreases with age. Complex alterations in the structure of collagen type III lead to decreasing dermal stability^[20]. Our study confirmed AFM upregulates COL I and COL III mRNA expressions which were associated with higher collagen deposition based on Sirius Red quantification. The ratio between COL I and COL III was also reduced in our study which showed similarity with younger age collagen content. This finding was reconfirmed by the quantification of COL III mRNA expression in UVB-induced NHDFs as shown in Figure 3B.

In adult human skin, types I and III collagen account of 80-85% and 10-15%, respectively. During skin formation, collagen types undergo fluctuations in relative ratio during aging^[21]. In the fetus, the type I/III collagen ratio is 1 and subsequently increases with age in adolescents, adult and elderly to 2.27, 2.46 and 2.97, respectively^[7]. The elevation of type I/III collagen mRNA expression ratio in aged skin is consistent with the results in this study as exhibited in Figure 3B. Even though the level of COL I and COL III mRNA expressions in AFM increased, the type I/III collagen mRNA expression ratio level was decreased. These findings indicate AFM stimulates collagen synthesis. Some limitations of this study are we did not quantify the ROS and other senescence markers such as Senescence Associated- β -Galactosidase (SA- β Gal), p21 or p16 but focused on downregulation of MMP-1 and increases in cell viability and collagen deposition.

Conclusion

In this study, we demonstrated the protective effects of AFM on UVB-irradiated human fibroblast culture through increasing cell viability, collagen deposition, COL I and COL III mRNA expressions along with reducing MMP-1 expression.

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Abbreviations

AFM	: Achatina Fulica Mucous
ECM	: Extracellular Matrix
MMP	: Matrix Metalloproteinase
NDHFs	: Normal Dermal Human Fibroblasts
PRP	: Platelet Rich Plasma
qPCR	: quantitative Polymerase Chain Reaction
ROS	: Reactive Oxygen Stress
UVB	: Ultra Violet-B

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Potential Conflicts of Interests

None

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